1974

THE EVALUATION OF ALDEHYDES AND OTHER DIFUNCTIONAL COMPOUNDS AS CROSS-LINKING AGENTS FOR COLLAGEN*

Summary

On hydrothermal denaturation both native and tanned collagen tend to become more elastic. Wiederhorn has pioneered a method of estimating the thermally stable cross-links in kangaroo tail tendon. This method depends on determination of stress-strain and volume fraction data on the swollen material and application of an equation derived for a solvated rubber.

When calculating the actual number of cross-links by the equation, a precise understanding of the terms involved is essential. In the amorphous network of collagen there will be half as many cross-links as chains providing that four chains meet in each cross-link.

The practical value of the technique has been demonstrated in the present comparative studies of tanning with difunctional aldehydes. Under optimum conditions glutaraldehyde and acrolein will insert more cross-links than either glyoxal or formaldehyde. The cross-links introduced by acrolein and glutaraldehyde are remarkably stable to the action of boiling water and to dilute acid at 20°C.

The marked effect of small amounts of chromium in retarding the deterioration of leather by moist heat has focused attention on the importance of cross-linking as a means of increasing the stability of leather[1]. To enable comparisons to be drawn both within and between the various groups of difunctional organic compounds capable of cross-linking collagen, it was necessary to select a relatively rapid method of estimating the number and the stability of the covalent cross-links introduced by a particular treatment. Although limited in that only bonds of sufficient stability to survive hydrothermal shrinkage are determined, the method developed by Wiederhorn and co-workers [2, 3] has met this requirement. The present intention is to comment

* The work reported forms part of a research programme sponsored by U.S. Department of Agriculture under the authority of U.S. Public Law 480. The paper is based on a lecture given at the Annual Conference of the Society of Leather Trades' Chemists, 1962.

on the theoretical interpretation of data derived by this method and to describe its practical application to collagen tanned with aldehydes.

Theoretical

The conception of cross-linking in relation to structures of high molecular weight has arisen in two related contexts. The term " cross-link " on the one hand is used to describe a lateral bridge between two long chains of atoms in an orientated high polymer, whilst on the other it is used to describe a junction point where four or more chain segments meet in the giant amorphous network of a vulcanised rubber or random synthetic high polymer.

In native collagen the most important type of cross-link stabilising the specific arrangement of the polypeptide chains is the hydrogen bond. During the conversion of skins and hides into leather by most tanning processes, it is believed that additional cross-links are introduced between these chains. The new bonds reinforce those already maintaining the secondary structure of the protein, thereby leading to some general modification in its properties and notably to increased hydrothermal stability. Now on raising the temperature of native or tanned collagen in the presence of water the net rate of hydrogen bond breakdown increases. At the shrinkage temperature there are no longer sufficient bonds to maintain the orientated structure and a random amorphous network tends to be formed in which the surviving cross-links become the junction points. Once hydrothermal shrinkage has taken place more water flows into the interstices between the disorganised protein chains and the swollen mass becomes rubber-like. On cooling it remains in this state over a considerable range of temperature spanning the original shrinkage temperature.

Flory and Rehner[4] have developed an equation based on the statistical theory of rubber-like elasticity[5] which defines the force-extension relationship for a swollen elastomer.

$$f = \frac{RTN_e}{V_o} V_2^{\frac{1}{3}} (\alpha - \alpha^{-2}) \quad \text{- - - - - - - - - (1)}$$

where f = the force per unit cross-sectional area of the swollen unstretched sample

R = the gas constant

T = the absolute temperature

N_e = the number of elastically effective chain segments

V_o = the volume of the network before swelling

V_2 = the volume fraction, i.e. $\frac{\text{volume of dry sample}}{\text{volume of wet sample}}$

α = the extension ratio.

In an amorphous network four chain segments commonly meet in each cross-link and the number of cross-links exerting stabilising influences on such a network will equal half the number of elastically effective chain segments. For a large network Flory[5] has deduced a relationship between the effective number (N_e) and the actual number (N) of chain segments that are cross-linked at both ends:—

$$N_e = N\left(1 - \frac{2M_c}{M}\right) \text{ - - - - - - - - - (2)}$$

where M_c = the mean molecular weight per cross-linked chain segment

M = the mean molecular weight before cross-linking.

Thus the smaller the fraction $2M_c/M$ the more closely does N_e approximate to N. Although the size of M in relation to the amorphous collagen network is open to speculation, it must be presumed a least equal to the mean molecular weight of the polypeptide chains in native collagen *i.e.* ca. 120,000 and may correspond to a larger aggregated unit. In the experimental work of the present paper, the assumption has been made that N_e and N may be equated both in tanned and untanned collagen. This assumption is supported by chemical estimates of the number of cross-links in the former case (see page 263), but in the latter case the possibility that N may be substantially greater than N_e must be borne in mind. When N_e is put equal to N, N_e/V_o in equation (1) may be replaced by ρM_c, where ρ is 1·3, the density of dry collagen[6].

$$f = \frac{RT\rho V_2^{\frac{1}{3}}}{M_c}(\alpha - \alpha^{-2}) \text{ - - - - - - - - - (3)}$$

Providing that the Flory - Rehner equation holds for hydrothermally denatured collagen, a graph of (f) against $(\alpha - \alpha^{-2})$ will be a straight line passing through the origin. From the gradient, $RT\rho V_2^{\frac{1}{3}}/M_c$, M_c can be calculated and the number of cross-links (gram-molar) in unit mass will be given by $(2M_c)^{-1}$.

Experimental Methods

Isolation of Tendons

Tendons, dissected out from a fresh kangaroo or wallaby tail, were immediately placed into a 5% solution of sodium chloride. This solution was changed four times over a period of twenty-four hours. After washing in running water they were dehydrated in three changes of acetone and soxhlet extracted with ether to remove the small amount of fatty material present. The tendons were stored in the air dry condition.

Determination of Cross-links

The tendons were thoroughly soaked back in cold water and then denatured by placing in water at 96 – 100°c for 30 sec. if untanned or 120 sec. if tanned. After the appropriate period further reaction was arrested by rapidly transferring the specimen into cold water.

The stress-strain cycle of a given tendon was determined by use of the extensiometer shown in figure 1. A 6 cm. length of tendon was mounted between the clamps A and B. After equilibration, in the relaxed state, for 2 hours in the water bath at the temperature of testing (usually 65°c), the clamp A was adjusted downwards until the specimen was just in tension and the vertical scale reading on the stand D noted. From the difference between this reading and that for the clamps just in contact, the unstressed length was calculated. The specimen was then extended by ca. 15 per cent of the unstressed length in six to eight equal steps at 3 minute intervals and was returned to the point of no stress in a similar manner. At each stage the corresponding equilibrium tension was recorded from the dial of the indicator unit of the

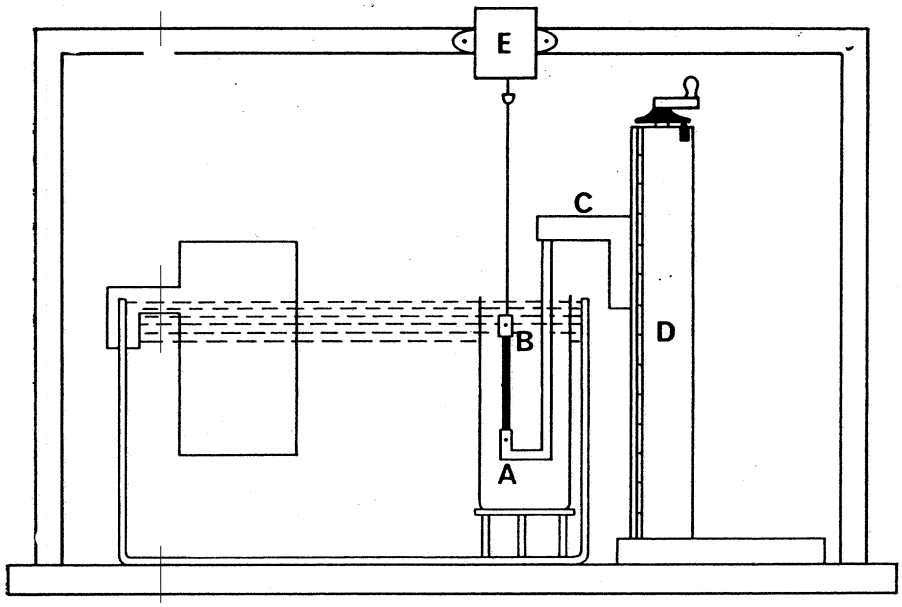


FIGURE 1

The Stress-Strain Extensiometer

The tendon is immersed in a thermostatically controlled water bath and is supported by clamps at A and B. Clamp A is fixed to an L-shaped bar which is rigidly fixed to the platform C of a heavy stand D. The platform may be moved vertically by rotation of a 0·5 cm. pitch screw and its height determined by reference to a scale graduated in mm. attached vertically to the upright of the stand D. A calibrated collar fixed to the screw enables small vertical displacements of the platform to be measured. The clamp B is attached by a wire to the strain-gauge transducer E.

strain-gauge transducer E. Finally the specimen was removed from the extensiometer by slicing through close to each clamp face with a sharp knife and the wet weight determined after lightly blotting. The wet volume at the temperature of testing was obtained by the specific gravity bottle method commonly used for an insoluble solid: —

$$\text{Volume} = \frac{\left\{\begin{matrix}\text{wt. bottle + water +} \\ \text{tendon (outside)}\end{matrix}\right\} - \left\{\begin{matrix}\text{wt. bottle + water +} \\ \text{tendon (inside)}\end{matrix}\right\}}{\text{density of water}}$$

From the volume and unstressed length the unstressed cross-sectional area was calculated and each equilibrium tension was expressed as (f), the force per unit unstressed cross-sectional area. Corresponding values of the function $(\alpha - \alpha^{-2})$ were calculated from the unstressed length and known extensions and were plotted against (f). The volume fraction (V_2) was calculated from the wet

weight and the " dry " weight obtained by acetone dehydration of the specimen followed by heating in air for 1 hour at 60°c, on the basis that: —

$$V_2 = \frac{\text{Vol of dry sample}}{\text{Vol of dry sample} + \text{Vol of swelling medium taken up}}$$

Then if W_o = weight of dry sample
W = weight of wet sample
ρ = density of dry collagen
ϕ = density of the swelling medium

$$V_2 = \frac{W_o\,\phi}{W\rho - W_o\,(\rho - \phi)}$$

Tanning with Water Soluble Difunctional Compounds

The acetone dehydrated tendon was first soaked back in water at room temperature. More usually tanning was carried out at 20°c for a period of 16 hours in an aqueous solution containing the difunctional compound, 10% w/v sodium sulphate and buffer salts in appropriate concentration to maintain the required pH Value. After tanning the tendon was normally well washed in water and dehydrated in three changes of acetone.

Results

Untanned Tendons

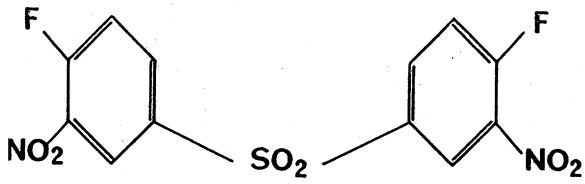
In the swollen denatured state freshly isolated kangaroo and wallaby tail tendons are elastic but begin to dissolve during the period of equilibration at 65°c prior to measurement of their stress-strain properties. Accurate determination of M_c is therefore difficult but from tests on fifteen specimens the value of 199,000 ± 15,000 has been assigned. On kangaroo tail tendon that had been commercially extracted for use as surgical sutures the value of M_c found was 55,000 in agreement with the result obtained on similar material by Wiederhorn and Reardon[3]. Wallaby tail tendon that had been acetone dehydrated and stored in the air-dry state for a number of years gave a value of M_c in the same region, viz. 64,000. The three values of M_c quoted, namely 199,000, 55,000 and 64,000 correspond to 1·0, 3·6 and 3·1 cross-links respectively per arbitrary unit of Mol. Wt. 400,000.

Tendons tanned with Difunctional Compounds

Reaction with pp'-Difluoro-mm'-dinitrodiphenyl Sulphone

The number of bridges introduced into tendon collagen by reaction with pp'-difluoro-mm'-dinitrodiphenyl sulphone (FF-Sulphone) have been determined chemically by Zahn and his colleagues. In the present investigation as an independent check of experimental methods and theoretical interpretation of results, tendons have been examined after reacting them with FF-Sulphone under analogous conditions.

Zahn and Wegerle[7] reacted a thin foil made from tendon collagen with this compound and after hydrolysis were able to isolate in yields accounting for about 60% of the reactive amino groups, three species of bridged amino-acid pairs namely that between lysine and lysine, hydroxylysine and hydroxyly-



sine and lysine and hydroxylysine. The reaction was further studied by Zahn and Nischwitz[8] using ^{14}C labelled FF-Sulphone for a series of reaction times.

In Table I the number of chemically estimated cross-links, calculated on the assumption that no intra-chain bridges were formed, are compared with the physical estimates of the present investigation and with those of Sykes[9]. Values of M_c before and after reaction with FF-Sulphone are quoted for each physical result.

TABLE I
Chemical and Physical Estimates of Cross-Links Introduced By FF-Sulphone

	Initial Mc	Short reaction time			Long reaction time		
		Time (hours)	Mc	Cross-links per unit of Mol.Wt. 100,000	Time (hours)	Mc	Cross-links per unit of Mol.Wt. 100,000
By Chemical Method (Zahn and Wegerle)					120		6·2
By Radio Chemical Method (Zahn and Nischwitz)		12		6·2	110		4·9
By Stress-Strain Method (Present Investigation)	199,000	16	17,400	2·6	120	8,000	6·0
By Stress-Strain Method (Sykes)	34,800	4	14,500	4·0	144	6,700	12·1

After long reaction, agreement between the chemical and the present physical estimate is satisfactory. Zahn and Nischwitz accounted for the lower yields of bridged amino-acid pairs resulting from extended reaction with excess FF-Sulphone by postulating the onset of a secondary reaction leading to the nucleophilic scission of some cross-links already formed and to unipoint fixation of FF-Sulphone at each amino-group. Because of greater surface

area and the smaller average distance that the reagent has to penetrate, both the primary and secondary reactions will proceed with the foil (thickness 0·015 mm) at a greater rate than with the tendon (diameter ca. 2 mm). It is therefore reasonable that the foil should contain more cross-links than the tendon after short times of reaction and vice versa for longer reaction times.

When allowance is made for the greater number of natural cross-links in the tendon that Sykes employed, his value of M_c after long reaction is consistent with that found in the present investigation. The greater number of cross-links reported is presumably related to a different interpretation of the connection between M_c and this value. It seems probable that the numbers of cross-links given by Sykes should be halved.

Tanning of Tendons with Aldehydes

Tendons have been tanned with aldehydes in amounts such that a considerable excess of free aldehyde was still present at the end of the tanning period. In Table II mean values of M_c and the numbers of cross-links per arbitrary unit of Mol. Wt. 100,000 are recorded for tendons tanned respectively with formaldehyde, glyoxal, glutaraldehyde and acrolein for 16 hours at 20°C at the given pH values. As the kangaroo tail tendon used for this series of experiments contained few natural, measurable cross-links the total number of cross-links determined does not differ to the nearest whole number from the number introduced by tannage.

TABLE II
Cross-Links in Tendons Tanned with Aldehydes in Excess

Aldehyde	pH of treatment	Mc	Number of Cross-links per Mol. Wt. 10^5	Uptake of Aldehyde Moles per 10^5g.
Untanned	—	200,000	0·2	—
Formaldehyde	8·0	8,900	6	80
Glyoxal	8·0	5,900	8	62
	8·0	5,600	9	170
Glutaraldehyde	6·5	3,900	13	94
	4·0	7,000	7	68
	8·0	4,500	11	129
Acrolein	6·0	9,000	6	—
	4·0	10,500	5	—

The uptakes of formaldehyde, glyoxal and glutaraldehyde were estimated by the loss of aldehydes from the tanning bath, that of acrolein at pH 8 from the weight increase of the tendon. Direct determination of formaldehyde fixed in skin under corresponding conditions, by the method of steam

distillation from dilute acid and precipitation with dimedone[10], gives values of 1 per cent or 30 moles per 10^5g. Thus in terms of protein reaction all the estimates are probably high. Even so, it seems likely that only a small proportion of the total aldehyde reacting participates in the formation of cross-links.

These results indicate that glutaraldehyde and acrolein are the more efficient cross-linking reagents in the group. The maximum number of cross-links is introduced by tannage with glutaraldehyde at pH 6·5. Probably at pH 4 reaction is restricted by the charge on the amino groups whereas at pH 8 the increased tendency of the aldehyde to polymerise may limit the desired reaction[11], but c.f. page 267.

Using similar conditions of formaldehyde tannage Wiederhorn *et al*[3] obtained the somewhat higher value for M_c of 15,000. By considering the cross-linking of one isolated protein chain segment of M_c equal to 55,000 in the untanned state, the insertion of 2·7 cross-links was inferred. However, the argument developed does not appear to take into account the second protein chain that must be involved in each cross-link. It is therefore probable that only half the stated number of cross-links may be credited to the given chain segment. On this new basis 2·5 cross-links would be introduced into a unit of Mol. Wt. 100,000 or approximately half the number found in the present investigation, (but see Table III).

In a further series of experiments tendon and skin pieces were treated together in equal volumes of solutions containing formaldehyde, glyoxal or glutaraldehyde at four levels such that the aldehyde concentration corresponded to:—

(a) 0·5 mole for each amino group in collagen
(b) 1·0 mole for each amino group in collagen
(c) 1·0 mole for each amino and guanidino (or amide) group in collagen
(d) 1·0 mole for each amino, guanidino and amide group in collagen.

Acrolein was included under conditions (a) and (d) only.

Glutaraldehyde was reacted at pH 6·5 and 8, the remainder at pH 8.

In Table III data related to the cross-linking of the tendons are recorded together with the shrinkage temperatures of the calf skin pieces. The formaldehyde treatments were on a batch of wallaby tail tendon which consistently gave higher than expected values of M_c irrespective of the cross-linking agent employed.

Assuming similarity of behaviour in skin and tendon it will be seen that there is no direct relationship between the number of cross-links introduced and the increase in shrinkage temperature. For glutaraldehyde and glyoxal shrinkage temperature reaches a maximum in concentration (c) while the number of cross-links continues to increase.

As with the first series of results glutaraldehyde forms more cross-links than either formaldehyde or glyoxal but in the range of concentration

TABLE III

Treatment of Tendons and Calf-Skin with Aldehydes in Stoichiometric Proportions

	Mc (tendon)	Number of cross-links in M.W. unit of 100,000	Moles of aldehydes offered for every cross-link formed (approx.)	T_s°C (calf skin)
Untanned	200,000	0·25	—	58
*Formaldehyde–pH 8				
(a)	26,800	2	9	76
(b)	22,000	2	15	81
(c)	15,500	3	28	85
(d)	15,400	3	45	87
Glyoxal–pH 8				
(a)	18,500	3	6	74
(b)	6,850	7	5	77
(c)	6,200	8	11	83
(d)	5,300	9	16	83
Glutaraldehyde–pH 8				
(a)	14,000	4	5	66
(b)	9,500	5	6	72
(c)	4,800	10	9	88
(d)	4,050	12	12	87
Glutaraldehyde–pH 6·5				
(a)	12,000	4	4	74
(b)	9,550	5	7	80
(c)	7,600	7	14	86
(d)	6,200	8	18	86
Acrolein–pH 8				
(a)	10,500	5	4	68
(d)	7,800	6	23	79

Amounts of aldehyde offered :
(a) 0·17 millimoles per g. of collagen = 0·5 moles per amino group
(b) 0·34 ,, ,, ,, ,, ,, = 1·0 ,, ,, ,, ,,
(c) 0·90 ,, ,, ,, ,, ,, = 1·0 ,, ,, ,, + amide or guanidino group
(d) 1·46 ,, ,, ,, ,, ,, = 1·0 ,, ,, ,, + amide + guanidino group

The ratio of air dry collagen to liquid was 1:20 w/v in each case.
*Wallaby tail tendon was used for this group of treatments, kangaroo tail tendon in the remainder.

covered by this experiment, tanning at pH 8 was more effective than at pH 6·5. More cross-links were also introduced at pH 8 than in the previous experiment with a large excess of aldehyde. A possible explanation is that at relatively low aldehyde concentrations cross-linking is to a lesser degree impeded by other reactions such as polymerisation. Alternatively a secondary nucleophilic reaction, analogous to that postulated by Zahn and Nischwitz[8], may, in the presence of excess aldehyde, cause scission of cross-links already formed earlier in the reaction.

Stabilities of Cross-links Introduced by Aldehydes

In order to gain more insight into the stabilities of the cross-links formed by reacting collagen with aldehydes, tendons were subjected to the prolonged action either of cold dilute acid or of boiling water. After the acid treatment the tendons were washed in water, denatured for the standard time of two minutes in boiling water and were then tested by the stress-strain method. The stress-strain properties of the specimens after extended periods in boiling water were measured after the usual period of equilibration at 65°C. The results are recorded in Table IV.

TABLE IV
Stabilities of Cross-Links Introduced by Aldehydes

Treatment	Cross-links in Mol. Wt. unit 100,000				
	Formaldehyde at pH 8	Glyoxal at pH 8	Glutaraldehyde at pH 6·5	Glutaraldehyde at pH 8	Acrolein at pH 8
Stability in water at 100°C					
Control—2 minutes	5	8	9	10	11
Boiled in water—					
1 hour	3	8	10	11	—
3 ,, ...	—	5	—	—	—
5 ,, ...	0·5	—	—	—	—
7 ,, ...	—	2	9	10	9
Stability in acid at 20°C					
Treated in N.H_2SO_4 for—					
0 hours (control)	5	8	9	10	11
2 ,, ...	3	3	7	9	7
24 ,, ...	2	—	—	—	—
120 ,, ...	2	4	7	7	7

On the basis of both tests glutaraldehydes and acrolein exhibited good stabilities. In the acid treatment about half the cross-links introduced by formaldehyde and glyoxal were lost. The cross-links introduced by glyoxal were rather unstable and those by formaldehyde markedly unstable to the prolonged action of boiling water.

Discussion

Both natural and chemically cross-linked kangaroo and wallaby tail tendons tend to rubber-like behaviour on hydrothermal denaturation. If the equilibrium tensions be measured as the tendon is extended or retracted in a stepwise manner and the appropriate functions of tension and length, namely (f) and $(\alpha - \alpha^{-2})$, be plotted, then the points form a pair of almost linear curves. The graph representing retraction in almost all cases cuts the $(\alpha - \alpha^{-2})$ axis in a small positive intercept due to permanent set in the tendon. Inspection of many curves in the present investigation has confirmed the

conclusion of Wiederhorn and Reardon[2] that denatured collagen when swollen with solvent, approximately conforms to an equation of the same general form as the Flory-Rehner equation:—

$$f = m(\alpha - \alpha^{-2}) + d$$

where m and d are constants and d is either zero or negative and small.

Providing that certain assumptions are made (page 263) there is reasonable agreement between stress-strain and chemical estimates of the number of cross-links introduced by reaction with FF-Sulphone. It would, therefore, seem that for moderate degrees of cross-linking the same meaning may be assigned to the constant (m) as in the Flory-Rehner equation, namely:—

$$m = RT\ NeV_2^{\frac{1}{3}}/Vo = RT\rho V_2^{\frac{1}{3}}/M_c$$

Generalisation from this particular case would be dangerous and no claim is made that collagen exactly obeys this form of the equation at other levels of cross-linking or after reaction with other difunctional compounds. However, the present estimates on aldehyde tanned tendons suggest that reliable comparative values of M_c or of the number of cross-links introduced, can be calculated on the assumption that the equation is true. Even for the few types where chemical determination is possible, if the tendency to intra-chain bridging is considerable it is probable that the stress-strain method gives a better indication of the actual inter-chain bridges present than do the much more time consuming chemical methods.

Some consideration has been given to shrinkage temperature as a criterion of cross-linking in chemically tanned collagen. As the cross-links introduced by tannage tend to reinforce the molecular structure, the shrinkage temperature is also raised. This simple, relatively rapid determination does therefore give an indication of the occurrence of cross-linking although it is not directly related to the number of cross-links introduced, being affected by many factors such as pH, the rate of heating and more particularly the solvent. Witnauer and Fee[12] have suggested that the decreased accessibility of the fibres as a consequence of tannage, contributes to the enhanced hydrothermal stability.

Freshly isolated kangaroo and wallaby tail tendons proved to have fewer measurable cross-links than the commercially extracted material originally examined by Wiederhorn and Reardon[2]. Possibly the difference between the two types of material is purely physical in nature, being due to a simple cause such as the sticking together of fibrils during drying, but the lower values of M_c found for acetone dehydrated wallaby tail tendon, after long storage in air, suggest that changes of a fundamental nature slowly take place after isolation from the animal. The decreasing solubility of precipitated acid soluble collagen on storage has been generally observed. It is possible that the in vitro changes taking place in both tendon and acid-soluble collagen are due to the formation of inter-chain cross-links.

The number of cross-links found in freshly isolated tendon would not account for all the ester linkages recently reported in collagen [13, 14]. However, bearing in mind that only covalent bonds in excess of the minimum number

required to combine the protein chains into giant networks are taken into account in the derivation of the Flory-Rehner equation[5], the actual number of cross-links must be significantly greater than the number determined. For this reason it is doubtful whether stress-strain studies on collagen in the rubber-like state can throw further light on the problem of the natural inter-chain linkages. Present primary concern was determination of the correction factor for natural cross-links to be applied in estimations on chemically modified collagen in order to determine the number of cross-links actually introduced by tannage

The practical value of the stress-strain method has been emphasised in a comparative assessment of the difunctional aldehydes as tanning agents for collagen. Not only can relative assessments be made of the numbers of cross-links introduced but differences in the stabilities of these bonds can be detected. Thus under optimum conditions glutaraldehyde is able to introduce more cross-links than the other three aldehydes tested, although acrolein is nearly as effective. Glyoxal is rather less effective but is better than formaldehyde as a cross-linking agent for collagen. In general it has been shown that in terms of the ratio of moles of aldehyde offered to cross-links formed, the reaction is most efficient when the ratio of aldehyde to collagen is low.

The action of moist heat is known to be the most important factor in the deterioration of leather in wear[15]. Any bonds which survive hydrothermal shrinkage clearly have a considerable resistance to heat and water but in an attempt to show differences between the stabilities of such bonds tendons treated with aldehydes have been subjected to periods of up to seven hours suspension in boiling water. Quite remarkable stabilities were exhibited by glutaraldehyde and acrolein. Glyoxal, too, was moderately stable but formaldehyde bonds were vulnerable.

It is feasible that the introduction of additional stable covalent cross-links in pelt before chrome tannage could impart new properties to the leather. Such cross-links would, of course, have to stand up to the pH conditions of tannage. For this reason tendons have been thoroughly extracted with 2N sulphuric acid at 20°C and the residual hydrothermally stable cross-links determined by the method described after first washing in cold water to remove the excess acid. Glutaraldehyde and acrolein bridges were shown to be quite stable to acid, those of glyoxal rather less stable and those of formaldehyde least stable.

The differences of stability found for glutaraldehyde, glyoxal and formaldehyde may possibly be explained by differences in their molecular lengths and in terms of the relative stabilities of bonds formed between the aldehyde and the amino, amide and guanidino groups of the collagen. Glutaraldehyde is presumed able to bridge the distances between amino groups and all the cross-links formed are of this type. In collagen there are thirty-four amino groups in a unit of Mol. Wt. 100,000 hence seventeen cross-links could be accommodated in this unit and the maximum number found by reaction with glutaraldehyde is thirteen.

With formaldehyde the primary reaction is the formation of the methylol derivative of collagen. Cross-linking is then believed to proceed via a secondary reaction involving either a pair of methylol amino groups or one methylol amino group and a free amide or guanidino group[16]. The net result is the insertion of a short cross-link consisting possibly of a single methylene group between the two reacting protein side chains. It is therefore hardly surprising that a smaller number of cross-links may be introduced with formaldehyde than with longer chain aldehydes. The liability of some of the formaldehyde cross-links to boiling water or dilute acid may be attributable to weakness when a particular side chain, such as amide or guanidino, is involved. Glyoxal can possibly form a bridge of sufficient length to span a few of the inter amino distances but some of the bonds involve other groups and are less stable. Combination between the aldehydic group and the amino group of collagen is suggested as the primary reaction with acrolein, with completion of cross-linking by reaction at the double bonds. The efficiency of cross-linking with this aldehyde, particularly at low concentration where the ratio of moles offered to cross-links formed is only 4: 1, goes some way to explaining the mechanism of chamois tannage. Presumably even the small amount of acrolein produced during the oxidation of cod oil is sufficient to form an appreciable number of stable cross-links.

Finally the question inevitably arises of how far are data obtained on tendon applicable to skin collagen. In principle direct determination of the number of cross-links introduced into hide by tannage might be accomplished either by the stress-strain method on denatured single fibres, or by swelling measurements on denatured hide in the form of powder and application of an equation developed for the swelling of rubber[17]. In practice neither method is straight forward. Difficulties with the former are mainly associated with the small size of the sample whilst in the latter it is necessary to evaluate an empirical constant in the equation by an independent method. However, some preliminary experiments carried out on formaldehyde tanned hide fibres by the author using both methods, do suggest that there are more naturally occurring, hydrothermally stable cross-links in hide and that under optimum conditions a somewhat larger number of cross-links may be introduced by tannage. It therefore seems a reasonable supposition that if a given difunctional compound is shown to cross-link tendon collagen efficiently, it will also be effective for skin collagen.

British Leather Manufacturers' Research Association,
Egham,
Surrey.

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